(36)

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/82, A01H 4/00 | A1

(11) International Publication Number: WO 97/43430

(43) International Publication Date: 20 November 1997 (20.11.97)

(21) International Application Number: PCT/US97/08242

(22) International Filing Date: 15 May 1997 (15.05.97)

(30) Priority Data:
08/648,775 16 May 1996 (16.05.96) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/648,775 (CIP)
Filed on 16 May 1996 (16.05.96)

(71) Applicant *(for all designated States except US)*: UNIVERSITY OF NORTH TEXAS [US/US]; Avenue C at Chestnut, P.O. Box 13646, Denton, TX 76203 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: CHAPMAN, Kent, D. [US/US]; 2125 Fairfax Road, Denton, TX 76205 (US). HEMPHILL, John, K. [US/US]; Apartment 3722, 1408 Teasley Lane, Denton, TX 76205 (US). MAIER, Camelia, G., A. [RO/US]; 716 Cherry Street Northwest, Ardmore, OK 73401 (US).

(74) Agents: HARRE, John, A. et al.; Sidley & Austin, Suite 4500, 1201 Elm Street, Dallas, TX 75270-2197 (US).

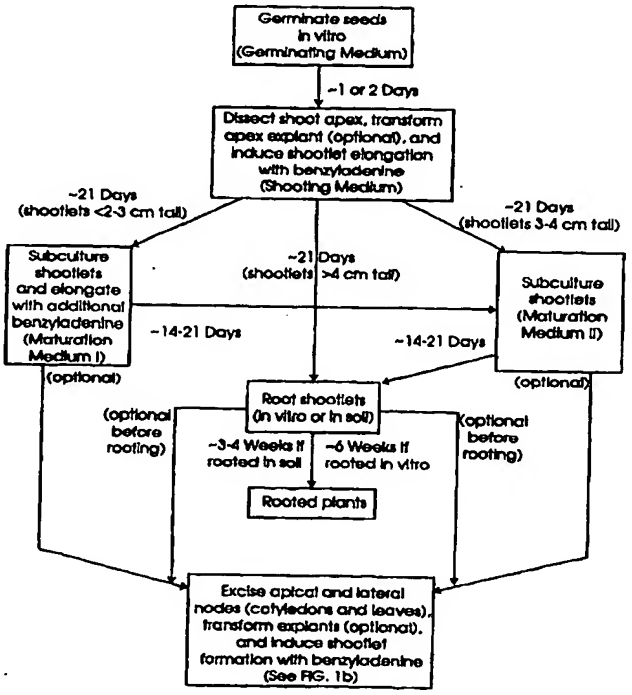
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: A RAPID *IN VITRO* REGENERATION SCHEME OF COTTON PLANTS COMPATIBLE WITH *AGROBACTERIUM*-MEDIATED TRANSFORMATION

(57) Abstract

This invention relates to a versatile method of rapidly regenerating cotton plants from explants of apical and/or nodal meristematic tissues which can be coupled with well known methods of transformation such as *Agrobacterium*-mediated transformation for the rapid production of genetically-engineered cotton varieties of agronomic importance.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

A RAPID *IN VITRO* REGENERATION SCHEME OF COTTON PLANTS COMPATIBLE WITH *AGROBACTERIUM*-MEDIATED TRANSFORMATION

TECHNICAL FIELD OF THE INVENTION

This invention relates to a method for regenerating whole cotton plants from transformed or nontransformed cotton tissues.

BACKGROUND OF THE INVENTION

Besides producing spinnable fibers, cotton (*Gossypium*) plants produce seeds with a potential multi-product base such as hulls, oil, linters and meal, each of which has a number of uses in a wide variety of markets. National Cottonseed Products Association, *Cottonseed and Its Products*, 2nd Edition, pp. 12-13 (1990). Per ton of seed crushed, cottonseed yields 540 lbs of hulls (27%), 320 lbs of crude oil (16%), 160 lbs of linters (8%) and 900 lbs of meal (45%). National Cottonseed Products Association, *Cottonseed and Its Products*, 2nd Edition, p. 16 (1990). These cottonseed products enter markets that are highly competitive. Under numerous marketing pressures, each product must stand on its merits or yield to its competitors that serve the consumer more effectively or at lower cost. This dynamic business environment dictates the requirement for a highly efficient regeneration/transformation system to effectively address changes within the marketplace. Therefore, there exists a need for a rapid, versatile regeneration/transformation system that targets cotton plants and which provides for the expression of seed specific added-value traits.

The art of plant tissue culture has been an area of active research for decades. Over the past 10 to 20 years, this scientific research has been intensified in an effort to develop regenerable plant tissue culture procedures not only for cotton but also for other important agricultural crops such as maize, wheat, rice, and soybeans. While the propagation of many of these agronomically significant crop plants *in vitro* is now commonplace, the routine culture and regeneration of cotton plants remains difficult. To date, a micropropagation system has not been defined for diverse cotton germlines which can be coupled with existing transgene technology. Therefore, the *in vitro* culture systems developed to date do not offer the cotton biotechnology industry with a versatile and rapid regeneration/transformation system that provides a means to move added-value seed traits into cotton.

One of the first cell culture regeneration systems for cotton was described by Davidonis and Hamilton. They successfully regenerated whole plants from somatic embryos of cotton. Davidonis, G. H. and R. H. Hamilton, *Plant Sci. Lett.* 32:89-93 (1984). These experimenters used immature cotyledon tissues of the cultivar Coker 310. The basic medium used consisted of Linsmaier and Skoog (LS) salts, vitamins, and

the growth regulators α-naphthaleneacetic acid (NAA) and kinetin. Linsmaier, E. M. and F. Skoog, *Physiol. Plant* 18:100-127 (1965).

Later that same year, the regeneration of plants from several different California cotton cultivars was reported. Rangan, T.S. et al., *In Vitro* 20:256 (1984). In this procedure, callus cultures were established from somatic tissues as well as immature embryos on Murashige and Skoog (MS) medium (Murashige, T. and F. Skoog, *Physiol. Plant.* 15:473-497 (1962)) supplemented with auxin or auxin and cytokinin. After the third to fifth subculture, these tissues gave rise to embryogenic callus and somatic embryos. The embryos were then transferred to a low salt medium, e.g., Beasley and Ting's (BT) medium (Beasley, C. A. and I. P. Ting, *Amer. J. Bot.* 60:130-139 (1973)) plus casein hydrolysate which permitted some of them to germinate and grow into whole plants. However, plants regenerated with this method exhibited significant amounts (30%) of sterility and genetic change by somaclonal variation. Stelly, et al., *Genome* 32:762-770 (1989).

Somatic embryogenesis was also observed using the Coker line 312 and a Texas race stock called T25. Robacker, D. C. and T. W. Zimmerman, *In the Ann. Mtg. of the American Society of Agronomy*, November 25-30, Las Vegas, Nev., p. 85 (1984). The basal medium consisted of MS salts, the vitamins inositol and thiamine, sucrose, and the growth regulators NAA, 2,4-dichlorophenoxyacetic acid (2,4-D), and kinetin. Hypocotyls were used as the original tissue source. Even though embryos were recovered and cultured onto BT medium, no plants were recovered.

Currently two cotton culture/regeneration systems have been reported. The first method involves the regeneration of cotton plants by somatic embryogenesis which relies on the induction of embryo formation from callus tissue as described above. The second method involves the recovery of plants by culturing the apices of shoots.

The currently utilized system for whole plant regeneration of cotton by somatic embryogenesis was first developed by Trolinder and coworkers in 1987. Trolinder, N. and J.R. Goodin, *Plant Cell Reports* 6:231-234 (1987); *see also* Firoozabady, et al., *Plant Molecular Biol.* 10:105-116 (1987). Unfortunately, while a wide range of cultivars were screened, only a few related varieties of cotton were amenable to this type of regeneration methodology. Moreover, none of these cultivars have agronomic

significance to today's farmers. Therefore, any genetic engineering projects employing this embryogenesis strategy with a Coker or other responsive line must incorporate an extensive 6-to 10-year conventional breeding program to transfer added-value gene traits into agronomically useful germplasm.

Furthermore, somatic embryogenesis typically requires relatively long periods in culture (8-12 months) which are labor intensive and contribute to infertility and genetic changes arising from somaclonal variation. Stelly, et al., *Genome* 32:762-770 (1989). More recently, there have been reports of the application of somatic embryogenesis to a broarder range of cotton lines. Firoozabady, E. and L. DeBoer, *In Vitro Cell Devel. Biol.* 29P:166-173 (1993). Koonce, L., and N.L. Trolinder, *Beltwide Cotton Conferences,* Nashville, Tennessee, January 8-12 (1996). However, the procedures do not avoid the limitations associated with somaclonal variation.

An alternative approach for whole plant regeneration - micropropagation via culturing of pre-existing apical meristems - was taken by Gould and co-workers which substantially decreased the time required for *in vitro* development of plantlets and also reduced the number of abnormal plants recovered. Gould, et al., *Plant Cell Reports,* 10:12-16 (1991). This methodology was based upon culturing existing shoot apices which were excised from 3 day old seedlings. No growth regulators were added to manipulate plant development. Similar results were noted by Bajaj and Gill where excised meristems (1 mm) and shoot tips (1 cm) of two *Gossypium* species (*G. arboreum* and *G. hirsutum*) were vegetatively regenerated with and/or without a callus phase. Bajaj, Y.P.S. and M.L. Gill, *Ind. J. Exp. Biol.* 24:581-583 (1986). In this study, kinetin (1, 2, 4 and 6 mg/l) plus indole acetic acid (IAA) (0.5 mg/l) was reported to support shootlet formation in both *Gossypium* sp. The authors also reported shootlet formation in the presence of a combination of benzyladenine (2 mg/l) and NAA (0.5 mg/l); however, no supporting data was provided. Neither of these clonal regeneration systems were successfully coupled with gene transfer.

In U.S. Patent 5,164,310 ("the '310 patent") there is reported a method for possibly transforming and regenerating the shoot apex of two cotton cultivars, *Gossypium hirsutum*, var. Coker 312 Tamcot CAB-C and *Gossypium barbadense*, var. Pima 5-6. However, no data was provided confirming GUS incorporation into the plant genome.

There is no disclosure in the '310 patent of the ability to transform and regenerate other than the shoot apices from 5 day old seedlings. In addition, the '310 teaches that regeneration should occur in the complete absence of hormones such as benzyladenine. The '310 patent also appears to be genotype dependent, i.e., it is limited to only the Coker and Pima cultivars. Thus, to transform other cultivars would still therefore require the extensive conventional breeding programs avoided by the present invention.

Transgenic cotton plants have been regenerated from embryogenic cells or calli derived from hypocotyls (Umbeck, et al., *Bio/Technology* 5:263 (1987); U.S. Patent No. 5,004,863) or cotyledonary tissue (Firoozabady, et al., *Plant Molecular Biol.* 10:105-116 (1987)) co-cultivated with *Agrobacterium tumefaciens*. Unfortunately, these methods have been successfully applied to only a few regenerable cotton cultivars (e.g., Coker 312). However, there are nearly one hundred cotton cultivars under cultivation in the United States, and they are not, in general, amenable to tissue culture techniques as described for Coker 312. Koonce, L., and N.L. Trolinder, *Beltwide Cotton Conferences*, Nashville, Tennessee, January 8-12 (1996); Firoozabady, E. and D.L. DeBoer, *In Vitro Cell Dev. Biol.* 29P:166-173 (1993).

As a result, the established technology used today for cotton is to: transform embryogenic cells of the Coker or other responsive lines; regenerate plantlets through somatic embryogenesis; collect T_1 seeds from T_0 plants; and advance the desired trait into an agronomic background by conventional plant breeding techniques. This methodology requires an additional 6 to 10 years with 2 to 3 crosses per year to transfer the added-value traits into the more agronomically superior cultivars. Moreover, plants regenerated from an embryogenic callus phase are often sterile and/or show signs of genetic change through somaclonal variation which affects both the phenotype and genotype of the plant. Firoozabady, E. and D.L. DeBoer, *In Vitro Cell Dev. Biol.* 29P:166-173 (1993); Stelly, D.M. et al., *Genome* 32:762-770 (1989); U.S. Patent No. 5,004,863. In addition, cotton plant regeneration through somatic embryogenesis remains germplasm-dependent. Koonce, L., and N.L. Trolinder, *Beltwide Cotton Conferences*, Nashville, Tennessee, January 8-12 (1996).

Thus, the cotton industry needs a rapid, reliable regeneration system that regenerates whole plants without a callus phase or somatic embryogenesis, and which

works with all germlines that are currently grown commercially in the field, breeder lines, experimental lines, and foreign germlines. Furthermore, the regeneration system must work with transformed plants, preferably *Agrobacterium*-mediated transformants. This system would bring significant advantages to the cotton industry. Emphasis on extensive conventional breeding techniques would be reduced, somatic embryogenesis and its limitations would be avoided, and finally, it would provide the means to directly incorporate genetic traits into today's commercial lines within a short period of time.

A number of regeneration or regeneration/transformation systems have been developed for plants other than cotton. However, none of these have been successfully applied to cotton. While some of these methods use benzyladenine ("BA") as a media supplement, there are no known reports of regenerating/culturing cotton using benzylamine as the sole growth regulator as is disclosed in the present application.

U.S. Patent No. 4,992,375 ("the '375 patent") is directed to a method of regenerating soybeans from cultured soybean cotyledonary nodes. Although the regeneration system disclosed in the '375 patent uses explants of cotyledonary nodes, the genetic requirements for whole plant regeneration from soybean cotyledons differ from those of cotton. The '375 patent also discloses that a critical feature of the system is the division of the node tissue into multiple pieces. By contrast, the subdivision of cotton nodes results in abnormal shoot formation. The system of the '375 patent also differs from the system disclosed herein for cotton in that it produces callus tissue, and it uses a benzyladenine concentration 5-50 times greater than that found necessary for optimum shootlet formation from cotton nodes.

U.S. Patent No. 5,416,011 and International Application No. PCT/US93/07009 are directed to methods for *Agrobacterium*-mediated transformation of soybean explants and regeneration using a cotyledonary regeneration system similar to that described in the '375 patent.

Other regeneration systems are described in Japanese Patent No. 5,176,647 which discloses a method of tissue culturing and mass propagating plants of the genus *Cytisus* and Japanese Patent No. 5,023,071, which discloses a method of proliferating adventitious buds of peony.

To date, none of these systems have been successfully adapted for use with cotton plants. Thus, there remains a need for a regeneration method that overcomes the current problems associated with the regeneration of cotton plants - long culture periods, somaclonal variation, infertility, and applicability to a limited number of cultivars. To that end, an *in vitro* culture and regeneration system for the rapid regeneration of the fertile cotton plants via micropropagation has now been developed. This regeneration system can also be used to regenerate whole cotton plants from genetically transformed cotton cells or tissues, allowing for the first time the rapid genetic engineering of improved commercial cotton varieties by clonal propagation. Finally, the systems disclosed herein can be used to regenerate and/or transform and regenerate dicotyledons other than cotton.

Using the regeneration system disclosed herein, elongated shootlets were induced to proliferate from explants of plant tissues comprising pre-existing meristems such as nodal or apical meristems on agar nutrient medium supplemented with a low concentration of benzyladenine. The resulting shootlets were then rooted with high efficiency. Regenerated plants of both glandless and glanded cotton varieties were in soil as early as six weeks after initiating cultures and matured plants that were advanced were all phenotypically normal and fertile. This regeneration system provides the capability to introduce genes directly into cultivars of commercially important varieties both rapidly and efficiently to produce cotton plants with added-value traits. As demonstrated, this regeneration system is compatible with existing transgene technology such as introduction of foreign DNA via *Agrobacterium* (Firoozabody, et al., *Plant Mol Biol.* 10:105-116 (1987)), and should also prove useful with other transgene technologies such as particle bombardment (Chlan, et al., *Plant Mol. Biol. Reporter* 13(1):31-37 (1995)).

SUMMARY OF THE INVENTION

The present invention involves the regeneration of whole cotton plants from explants of plant tissues comprising pre-existing meristems such as nodal or apical meristematic tissues.

In another embodiment, the present invention involves the transformation of explants of nodal or apical meristematic tissues and the subsequent regeneration of these transformed tissues into whole cotton plants. In this manner, the present invention is useful in the rapid genetic engineering of cotton plants in general and of the commercially important cultivars in particular.

In other embodiments, this invention relates to cotton plants produced using the procedures disclosed herein, seeds produced from these plants, and cotton plants germinated from these seeds.

In still other embodiments, the invention relates to the regeneration or the transformation and regeneration of dicots other than cotton.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1a and 1b are exemplary schematic representations of the preferred regeneration/transformation systems disclosed herein.

DETAILED DESCRIPTION

A rapid, direct clonal propagation system has been developed to regenerate plants, preferably mature cotton (*Gossypium hirsutum L.*) plants, from explants of plant tissues comprising pre-existing meristematic tissues including the nodal and apical meristems of *in vitro* grown cotton seedlings or plants. (Figs. 1a and 1b). This system has been found to be useful with a wide variety of cultivars (both glanded and glandless) including Stoneville 7A (glandless), Stoneville 474 (glanded), Paymaster HS-26 (glanded), CA-3050 (glanded), CA-3066 (glanded), CA-3076 (glanded), CA-3084 (glanded), and Stovepipe (glanded) (Table 3). The Stoneville 7A seeds were provided by Dr. Rick B. Turley, USDA-ARS, Stoneville, MS. The Paymaster HS-26 seeds were provided by Dr. John J. Burke, USDA-ARS, Lubbock, Texas. The CA-series and Stovepipe seeds were provided by Dr. John Gannaway, Texas Agricultural Experiment Station, the Texas A&M University System, Lubbock, Texas, and the Stoneville 474 seeds were provided by Dr. Catherine Houck, Calgene, Davis, California.

This clonal propagation system preferably utilizes different explants isolated as described in Examples 1 and 2 by excising apical (Fig. 1a) and/or nodal (Fig. 1b) meristematic tissue from cotton seedlings (or plants) or germinated seeds germinated and grown under aseptic conditions or from clonally propagated shootlets ("CPS") grown under aseptic conditions. (Figs. 1a and 1b). Preferably, nodal meristematic tissues, i.e., leaf nodes, including primary leaf nodes, and/or cotyledonary nodes are excised from cotton seedlings or plants having an apical and at least 4 lateral or leaf nodes. The period of growth needed to reach this stage depends on the seed vigor but typically takes less than or about 28 days. For plants derived from seeds with greater seed vigor, nodal expants may be taken from seedlings or plants 14 to 28 days old, and even from seedlings or plants 14 to 21 days old. Although cotton seedlings or plants of the ages described above are preferred, nodal meristematic tissue can be isolated from any plant bearing the appropriate tissues.

Apical meristematic tissue is preferably excised from seedlings or plants less than about 28 days old, more preferably from seedlings or plants less than about 21 days old, even more preferably from seedlings or plants less than about 14 days old and most preferably from germinated seeds about 1 or 2 days old. As before, although cotton

plants of the ages described above are preferred, apical meristematic tissue can be isolated from any plant bearing such tissue at any time after the radicle breaks the seed coat.

Once isolated, the explants are placed vertically (basal end down) on a solid Murashige and Skoog ("MS") nutrient medium, Murashige, T. and F. Skoog, *Physiol. Plant.* 15:473-497 (1962) (incorporated herein by reference), supplemented with a carbon source, preferably sucrose, and a low concentration of cytokinin, preferably benzyladenine (BA). The supplemented medium as described above is hereinafter referred to as the Shooting Medium. (Table 1). Meristematic explants from seedlings or plants preferably less than about 28 days old can be cultured on the Shooting Medium and regenerate elongated shootlets directly without callus formation suitable for rooting generally in about 21 days.

The concentration of BA found to be effective in producing elongated shootlets, i.e., shootlets greater than 2-3 cm in about 21 days, without callus formation from explants of nodal meristematic tissue is less than about 1 μM, preferably from about 0.15 μM to about 1.0 μM, even more preferably from about 0.3 μM to about 0.5 μM, and most preferably about 0.3 μM. Optimum formation of elongated shootlets from explants of the apical meristematic tissue from seedlings or plants is obtained with about 1.0 μM whereas optimum formation of elongated shootlets from explants of the apical meristematic tissue from germinating seeds is obtained with about 0 to about 1.0 μM BA. In the absence of BA, shooting has been observed for some cultivars; however, formation of elongated shootlets is neither consistent nor reproducable for explants other than those comprising apices isolated from 1 to 2-day old seedlings. Concentrations of BA higher than about 1.0 μM are to be avoided as they are increasingly toxic to the explants.

After an approximately 3 week culture period, multiple shootlets (2-5) can be proliferated from nodal explants cultured on the Shooting Medium containing BA, preferably 0.3 μM. Furthermore, new shootlets can continue to proliferate from these explants after elongated shootlets are harvested. In addition, shootlets can be proliferated from excised nodes, i.e., explants, of the elongated shootlets induced to proliferate *in vitro*. This provides a means to multiply the germline by clonal

propagation. Other explants, including cotyledon pieces, leaf pieces, epicotyl segments and hypocotyl segments produce callus at the same concentrations of BA but do not produce shootlets. (Table 2).

After shootlet proliferation, i.e., shootlet elongation from nodal or apical meristems, the shootlets are matured and transferred to an appropriate medium to foster or induce root formation preferably as described in Example 3. Shootlets less than about 2-3 cm tall after about 21 days are transferred to Maturation Medium I (Table 1) for 14-21 days to induce additional growth and to remove any remaining phenolic compounds prior to rooting and are then transferred to Maturation Medium II for an additional 14-21 days. Shootlets about 3-4 cm tall after about 21 days are transferred to Maturation Medium II (Table 1) to remove any remaining phenolic compounds prior to rooting. Shootlets greater than about 4 cm tall after about 21 days can be rooted directly without a maturation step. At the end of the maturation period of 14-21 days, the shootlets are ready for rooting, either *in vitro* or directly in soil. For *in vitro* rooting, a preferred Rooting Medium is MS media supplemented with about 1.0 μM indole-3-butyric acid (IBA) (Table 1). It has been found that shootlets treated in this manner typically form roots in about 6 weeks, after which the plantlets are transferred to soil, typically in 3 inch pots. Preferably, the shootlets are transferred directly to soil, e.g., in 3 inch pots after application of naphthalene acetamide to the bottom of the stem to induce rooting. Naphthalene acetamide is available commercially as Rootone™ powder (Greenlight Co., San Antonio, Texas). Shootlets treated in this manner typically form roots in about 2 to 3 weeks. Preferably, transgenic shootlets are rooted by transferring the shootlet directly to soil after the application of Rootone™. It has also been found that shootlets greater than about 3 cm tall will root more quickly (about 2 weeks) than shootlets less than about 3 cm tall (about 3 weeks) when rooted.

As shown in Table 11 (Example 4), the efficiency of rooting for cultivars was low *in vitro* (22-26% of the shootlets rooted). By contrast, rooting efficiency was greatly increased when shootlets were transferred directly to soil (50-90% of the shootlets rooted). Regenerated plants that were advanced to soil appeared morphologically similar to seed-borne cotton plants and showed no obvious chromosomal abnormalities. Furthermore, these regenerants flowered, produced bolls,

and set viable seeds. Other high-fiber-yielding breeder lines (cvs., CA-3066, ST474, CA-3076, CA-3084, CA-3050, and Stovepipe) were also found to be adaptable to this regeneration scheme. Using the *in vitro* propagation techniques disclosed herein, high-fiber-yielding varieties of cotton plants can be regenerated rapidly (explant-to-soil in approximately 6 weeks).

During or after the rooting process, the shootlets, which are rootless, or plantlets (rooted shootlets) are preferably exposed to a hardening process. Most preferably, the hardening process begins simultaneously with the initiation of the rooting process. In general, the hardening process takes approximately 2 to 3 weeks depending on the height of the elongated shootlets at the time the process is begun. Generally, the potted shootlets/plantlets are initially enclosed, preferably within plastic bags to generate the humid environment necessary for hardening purposes. The shootlets/plantlets are preferably nurtured for about 2 weeks in a humid environment with nutritional supplements. The cotton shootlets/plantlets are uncovered daily to add water. A nutritional water solution, preferably Miracle Gro™ (0.75 g/gallon) is added every third day. (Stern's Miracle Grow, Port Washington, N.Y.). The shootlets/plantlets are gradually removed from within the enclosed bags in stages over a 2 week period to allow for continual adaptation and plantlet growth. Preferably by the end of the third week, the plastic bags are completely removed. Smaller shootlets (approximately 2 cm in height) that are advanced to soil early usually require a longer exposure to the humid environment for root/plantlet formation and the hardening process. After root formation, the plantlets begin to elongate and develop new leaflets. Plantlets which produce new leaves during this hardening process are transplanted into larger, typcially 6 to 10 inch pots and grown in a greenroom until flowering and boll set.

The regeneration system described above can also be used to regenerate plants from transformed cotton explants comprising pre-existing meristems or other cotton tissues. The preferred transformation technique of the present invention makes use of the Ti plasmid of *Agrobacterium tumefacians*. The Ti plasmid has the natural ability to transfer a segment of itself, referred to as the transfer DNA (T-DNA) region, into the genome of infected plant cells. By inserting a foreign gene construction into the T-DNA region, the *Agrobacterium tumefaciens* can transform plant cells with the foreign gene.

The foreign gene construction is then included in the cells of a whole plant regenerated from the transformed cells and is then inherited in a simple Mendelian manner. The construction can thus be treated as any inheritable trait for crop breeding purposes.

Transformation can be performed with either the apical or nodal meristematic explants. Explants are transformed by co-cultivating the apical or nodal meristematic tissue with *Agrobacterium tumefaciens* strain LBA4404 harboring the binary vector pBI121 preferably as described in Example 5. While the *Agrobacterium tumefaciens* strain LBA4404 harboring the binary vector pBI121 is preferred for transformation, other vectors or *Agrobacterium* strains known to those of skill in the art can be used. In addition, other transformation procedures known to those of skill in the art can be used. The pBI121 vector carries both a selectable marker, i.e., the NPT II gene, for kanamycin resistance, and a GUS-reporter gene within the left and right borders of the T-DNA region. (Clontech Laboratories, Inc., Palo Alto, CA). Following the co-cultivation period, the explants are sequentially transferred to Shooting Medium supplemented with increasing amounts of kanamycin ("KAN") - KAN Selection Media - to select for transformed shootlets preferably as described in Examples 6 and 7. While selection using kanamycin resistance is preferred, insertion of gene sequences coding for resistance to other antibiotics such as neomycin, hygromycin, or chloramphenical or to other selectable genes known to those skilled in the art can be used.

Following co-cultivation of explants from 1 or 2-day old germinating seeds, the shootlets obtained exhibit three phenotypes during kanamycin selection - green, mottled green, or tan. The green phenotype is exhibitied by early emerging shootlets, generally evident after about five days and showing no signs of phenotypic stress, i.e., having green leaves and stems. The mottled green phenotype is exhibited by later emerging shootlets, generally evident after about 5 days to 2 weeks and showing various degrees of stress such as bleached leaves, white leaf tips and edges, and green sectors and white vascular tissue in cotyledonary leaves. The third phenotype, i.e., the tan phenotype, is exhibited by kanamycin-sensitive (non-transformed) explants which turn brown and die.

The mottled green phenotype grows more slowly during the KAN selection procedure which involves a step-wise incremental increase of the KAN concentration (37 to 50 to 75 μg/ml) to which the putative transformants are exposed. However,

newly formed leaves of the mottled green phenotype which emerge after about 2 weeks, are phenotypically normal. With both the green or mottled green phenotypes, the KAN-resistant shootlets should be carefully moved through the above-described KAN selection system. Essential to this success is the use of the cut/trim method in which the shootlets are cut and the cotyledonary leaves, and other lateral nodes trimmed or removed as necessary such that the apices of the shootlets are constantly less than about 2 cm from the antibiotic source. If desired, the excised cotyledonary and other lateral nodes can be clonally propagated. To maintain the apices of the elongating shootlets within about 2 cm of the source of antibiotics, the shootlets are evaluated on a daily basis to determine their height. For apical meristems, transfer to media with 50 μg/ml KAN (Kan Selection Medium II) from that with 37 μg/ml KAN (KAN Selection Medium I) and the cut/trim step is generally first required after about 4-6 days. For nodal meristems, transfer to KAN Selection Medium II from KAN Selection Medium I and if necessary the cut/trim step is generally first required after about 7 days. In either case, KAN selection is enhanced by keeping the meristem close to the antibiotic source and the vascular tissues of the shootlet in direct contact with the kanamycin supplemented medium. Following KAN selection, both the green and mottled green phenotypes are matured and rooted by the methods previously described herein with respect to the non-transformed explants.

Following co-cultivation of explants from the 14 to 28-day old seedlings and clonally propagated shootlets, the explants first become necrotic and then develop a green shootlet after about 7 to 10 days of the KAN selection process. As the shootlets grew and elongated, the necrotic explants were excised (in some cases - recultured) and the green shootlets were transferred to the fresh kanamycin medium. In general, the explants from the established shootlets were more sensitive to the kanamycin medium than explants from the 28-day old seedlings.

It is generally believed that the transformed (T_0) plants are chimerically transformed, i.e., some of the pre-existing meristems are transformed and others are not. To verify kanamycin resistance/transformation or kanamycin sensitivity/non-transformation, shootlets can be rechallenged on kanamycin rather than rooted. This is done by excising nodal or apical meristematic explants from these shootlets and

culturing them on fresh kanamycin supplemented medium to identify transgenic meristems within the shootlets. Successful transformation can also be verified by GUS enzymatic activity in the leaves of KAN selected plantlets as described in Example 9. (See Table 15). Stable or germline transformation can be verified by GUS enzymatic activity in the pollen grains (Tables 16-19) of the transformed plants as described in Example 10 or by Southern-blot analysis as described in Example 12.

Examples

Example 1 - Seed Sterilization and Germination

Seeds from diverse germplasm (Table 3) were sterilized, germinated, and grown *in vitro* for different time periods. For seed sterilization, seeds (approx. 150) were placed in ultrapure water (MilliQ plus UF) with two drops (approximately 100 μl) of Tween-20 per 100 ml of H_2O and washed with a brush gently for several minutes. This procedure was repeated until no more bubbles arose from the seeds during brushing, but in no case less than three times. The seeds were then wrapped in cheesecloth and submerged in running DI-water for about 3 hours. Next, the seeds were placed under sterile conditions in 70% ethanol containing Tween-20 (2 drops per 100 ml) for 60 seconds. The seeds were then collected in a sterile strainer and rinsed with sterile ultrapure water *(MilliQ plus UF)* for 3 minutes and placed in sterile 20% commercial bleach plus Tween-20 (2 drops per 100 ml) for 20 minutes with continuous mixing. The seeds were then thoroughly rinsed (3X) with sterile ultrapure water *(Milli-Q plus UF)* for 3 minutes. Following the rinsing step, the seeds were allowed to imbibe water until needed for experimentation. Approximately 4 hours was required for the complete sterilization/imbibition period.

For seed germination, seeds were placed on sterile filter paper moistened with 2 ml of water (*MilliQ plus UF*) within a Petri plate and allowed to germinate in the dark (30°C) overnight. The following day, the seed coats were removed and the shoot apex was isolated as described in Example 2 and placed directly on Shooting Medium (Fig. 1a). In some cases, the shoot apex was isolated after two days of germination in the dark rather than one depending on the seed vigor and the rate of germination.

Alternatively, the germinated seeds (minus seed coat) were placed on Germination Medium (Table 1) in Magenta vessels and allowed to grow under tissue culture chamber conditions (30°C; light intensity 85 μmol $s^{-1}m^{-2}$) for about 14 to 28 days. (Fig. 1b). To allow for seedling maturation, inverted sterile Magenta boxes and couplers were added to the existing seedling vessels. After the 14- to 28-day period, the cotton seedlings of the cultivars, containing 3 to 5 nodal meristems, had grown to approximately 7 to 10 inches in height. The following explants were then excised from the 14- to 28-day old seedlings: apical meristems and/or nodal meristems including, primary leaf nodes and cotyledonary nodes. These explants were harvested, placed directly on Shooting Medium (Table 1) and utilized as described herein.

Table 1. Tissue culture media utilized to regenerate and transform cotton plantlets.

Stage	Medium	Composition	Days or Hours
Seed Germination	Germination Medium	½ MS medium[a] with sucrose (10 g/l), Phytagel (2.25 g/l), (pH 7.0)	14-28
Shootlet Induction	Shooting Medium	MS medium with BA	21
Shootlet Maturation	Maturation Medium I	MS medium with BA and 3 g/l AC	14-21
	Maturation Medium II	MS Medium with 3 g/l AC	14-21
Root Formation	Rooting Medium	MS medium with 1 μM/l IBA	28-42
Co-cultivation Period	Co-cultivation Medium	MS medium with Sucrose 1.5 g/100 ml; Acetosyringone (20 mM Stock) 200 μl; MES (20 mM Stock) 10 ml; pH 5.5; Sterilization by membrane filtration (0.22 μ)	1 h
Induction/KAN-Selection Stage 1	KAN Selection Medium 1	MS Medium with 0.3 μM BA; 37 μg/ml Kanamycin; and 500 μg/ml Carbenicillin	5-7
KAN-Selection Stage 2	KAN-Selection Medium 2	MS Medium with 0.3 μM BA; 50 μg/ml Kanamycin; and 500 μg/ml Carbenicillin	28
KAN-Selection Stage 3	KAN-Selection Medium 3	MS Medium with 0.3 μM BA; 75 μg/ml Kanamycin; and 500 μg/ml Carbenicillin	28

Abbrev.: BA = β-benzyladenine; IBA = Indole-3-butyric Acid; AC = Activated Charcoal; MES = 2-(4-Morpholino)-Ethane Sulfonic Acid; KAN = Kanamycin.

[a]MS medium = Murashige & Skoog major & minor salts; MS vitamins; sucrose 15 g/l; Phytagel (Sigma) 2.2 g/l; pH 6.0 (except as otherwise noted).

Example 2 - Source Materials

For experiments utilizing tissue from 1 or 2-day old seedlings, seeds were surfaced sterilized by the procedure described in Example 1. Following the 1 or 2-day germination period, seeds that supported extended radicle growth (2 mm to 5 mm) were selected and their embryonic axes and portions of the attached cotyledons (approximately 3 to 4 mm in length) were isolated. The apices (approximately 2 mm to 3 mm in length) were placed vertically on the Shooting Medium (Table 1) in the same orientation as in the intact seedling. (Table 2).

For experiments utilizing tissue from *in vitro* grown 14- to 28-day old seedlings, the following explants were isolated: shoot apices; primary leaf nodes; leaf nodes; and cotyledonary nodes. The excised explants were placed vertically on the Shooting Medium (Table 1) and in the same orientation as in the intact seedling. (Table 2).

For experiments utilizing pre-existing meristems from clonally propagated shootlets, apices and lateral meristems were isolated and placed vertically on the shootlet induction media and in the same orientation as in the intact shootlet. These clonally propagated shootlets which were derived from diverse germlines (HS-26, CA-3076, ST7A, ST474 and ST139) were maintained for several months. While cultured on MS medium with 0.3 μM BA, the established shootlets could only be cultured for only about 3 weeks. The substitution of different vitamin sources for the MS vitamins improved the shootlets culturability in that they could be maintained for 5 to 6 weeks before being transferred to fresh medium. The other vitamin sources were as follows: (1) B5 vitamins (*Exp. Cell Res.* 50:151 (1968)) and (2) modified Nitsch's vitamins (*Science* 163:85 (1969); Thiamine-HCl at 10 mg/l and nicotinic acid at 0.5 mg/l). (Table 2).

Table 2. Comparison of Explant Sources Derived from *in vitro* Grown Cotton (Stoneville 7A, Glandless) Seedlings for Their Capacity to Form Shootlets When Cultured on Shooting Medium Containing 0.3 μM BA

Explant Sources	Response
Cotyledon pieces	Callus
Foliar leaves	Callus
Hypocotyl segments	Callus
Epicotyl segments	Callus
Cotyledonary nodes	Shootlets
Primary leaf nodes	Shootlets
Apices	Shootlets
CPS nodes*	Shootlets

*CPS nodes refers to nodes of clonally propagated shootlets.

Example 3 - Regeneration Procedures

Explants of the three different source materials were placed on the benzyladenine (BA) containing Shooting Medium to induce regeneration, i.e., shootlet proliferation. For apical meristematic tissue from 1 or 2-day old seedlings a benzyladenine concentration ranging from about 0 to about 1.0 μM was used. For apical meristematic explants from 14 to 28-day old seedlings a concentration of about 1.0 μM BA gave optimum results. For nodal meristemtic tissue from 14 to 28-day old seedlings and for meristematic tissue from clonally propagated shootlets a BA concentration of less than about 1 μM, preferably about 0.15 μM to about 1.0 μM, even more preferably about 0.3 μM to about 0.5 μM, and most preferably about 0.3 μM was used. The BA was added to the Shooting Medium immediately before autoclaving.

Following the proliferation of shootlets, a shootlet maturation step was used before rooting in most cases. Shootlets less than about 2-3 cm tall after 21 days were matured by transferring the explant to Maturation Medium I (Table 1) for an additional period of 14 to 21 days and then transferred to Maturation Medium II for an additional

14 to 21 days. Shootlets about 3-4 cm tall after 21 days were matured by transferring the explant to Maturation Medium II (Table 1) for an additional period of 14 to 21 days. Shootlets greater than about 4 cm tall after 21 days were not matured; but rather, were rooted immediately. In all cases, rooting was accomplished as described in Example 4. For those shootlets rooted *in vitro*, a Rooting Medium (Table 1) consisting of 1μM indole-3-butyric acid (IBA) substituted for benzyladenine in the Shooting Medium was used. The IBA was added to the Rooting Medium before autoclaving. Except when otherwise noted, the pH of media used was adjusted to 6.0 with NaOH(1N) or HCl(1N) prior to adding Phytagel and the media autoclaved at 1.46 kg/cm^2 for 15 minutes. Petri dishes (100 x 25 mm) were sealed with Parafilm and Magenta vessels were capped or enclosed with polypropylene covers. All cultures were maintained at a constant light intensity (85 μmol s^{-1}m^{-2}), temperature (30°C), and a 16 hr photoperiod. The light source consisted of cool white fluorescent lamps.

For rooting and initial plantlet development, the regenerants (nontransformed shootlets) and KAN selected (putatively transformed) shootlets were placed in a growth chamber which maintained controlled environmental conditions (temperature, 30°C; light intensity, 155 μmol s^{-1}m^{-2}; and a 16 hr photoperiod). The light source consisted of cool white fluorescent and incandescent lamps. For further plant growth and flowering, these plants were moved to a greenroom which supported continual plant growth and flowering under the following environmental conditions: high temperature (90-100°F) to low temperature (65-75°F); light intensity dependent upon the location within the room (66 to 134 μmol s^{-1}m^{-2}) and a 16 hr photoperiod. The light source consisted of high-pressure sodium (140 μmol s^{-1}m^{-2}) and metal halite lamps (100 μmol s^{-1}m^{-2}).

Example 4 - Plant Regeneration Results

The regeneration method disclosed herein was found to stimulate shootlet proliferation in a variety of diverse germplasms or cultivars. (Table 3). The method described herein for cotton plant regeneration was first observed in a preliminary experiment where BA was tested for its capability to generate shootlets from excised cotyledonary nodes of 14-day old seedlings (Stoneville 7A)(Table 4). Benzyladenine at 0.3 μM, when applied to the explants as part of the Shooting Medium, initiated

elongated shootlets from excised cotyledonary nodes after a 3 week culture period. In contrast, higher concentrations of BA (3.0 μM and greater) suppressed shootlet formation after a 3 week culture period and were often toxic to the explants. (Table 4).

Table 3. Shootlet Proliferation of Explants Excised from *in vitro* Grown Seedlings of Different Cotton (*Gossypium hirsutum*) Cultivars

Cultivars	Shootlet Formation
Stoneville 7A[a]	+[e]
Paymaster HS-26[b]	+
CA-3050[c]	+
CA-3066[c]	+
CA-3076[c]	+
CA-3084[c]	+
Stovepipe[c]	+
Stoneville 474[d]	+

[a]Stoneville 7A seeds were provided by Dr. Rick B. Turley, USDA-ARS, Stoneville, MS.

[b]Paymaster HS-26 seeds were provided by Dr. John J. Burke, USDA-ARS, Lubbock, TX.

[c] CA-Series and Stovepipe seeds were provided by Dr. John Gannaway, Texas Agricultural Experiment Station, The Texas A & M University System, Lubbock, TX.

[d] Stoneville 474 seeds were provided by Dr. Catherine Houck, Calgene, Davis, CA.

[e]"+" sign indicates formation of elongated shootlets from explants.

Table 4. Shootlet Proliferation from Excised Cotyledonary Nodes of 14-day old Cotton Seedlings (Stoneville 7A, Glandless) When Cultured on Shooting Medium Containing Different Concentrations of Benzyladenine (BA)

	BA Concentration (μM)				
Culture period	0.3	1.0	3.0	10.0	30.0
Week 2	4 [a]	2	3	0	0
Week 3	7	3	0	0	0

[a] The number of shootlets / 3 explants.

A range of BA concentrations (0.15 to 1.0 μM) when added to the Shooting Medium supported shootlet formation in excised primary leaf nodes and cotyledonary nodes isolated from 14- to 21-day old cotton seedlings (Stoneville 7A). (Table 5). The optimal BA concentrations for inducing shootlet formation from nodal meristems cultured on Shooting Medium was 0.3 μM based on the development of elongated shootlets (2-3 cm in height) after a 3 week culture period (Tables 5, 6 and 7). At 1.0 μM BA, the apical meristems isolated from 14 to 21-day old cotton seedlings also were optimally induced to form elongated shootlets (Tables 8 and 9). For apical meristems isolated from 1 or 2-day old germinated seeds a BA concentration of 0 to 1.0 μM was optimal. In contrast, hormone-free medium failed to support elongated shootlets of apices excised from seedlings other than the 1 to 2-day old germinated seeds during the same culture period in a consistent or reproducible manner. Similar results on shooting efficiencies and BA concentrations were found for the glanded variety, Paymaster HS-26 (Table 6) and the other cultivars used in the study (Table 7).

Table 5. Effect of Benzyladenine on Shooting Efficiencies of the Primary Leaf Nodes and the Cotyledonary Nodes Excised from 14- to 21-Day Old Seedlings of Cotton (Stoneville 7A, Glandless)

	Benzyladenine (μM)				
Culture period	0.0	0.15	0.3	0.5	1.0
Week 2	3/9[a]	14/17	18/24	12/15	5/8
Week 3	5/9	15/17	19/24	15/15	7/8

[a]The number of shootlets / number of nodal meristems.

Table 6. Effect of Benzyladenine on Shooting Efficiencies of the Primary Leaf Nodes and the Cotyledonary Nodes Excised from 14- to 21-Day Old Seedlings of Cotton (Paymaster HS-26).

	Benzyladenine (μM)					
Culture period	0.0	0.15	0.3	0.5	1.0	10.0
Week 2	3/35[a]	16/22	29/35	23/26	19/28	1/13
Week 3	16/35	15/22	29/35	24/26	22/28	0/13

[a]The number of shootlets / number of nodal meristems.

Table 7. Effect of Benzyladenine on shootlet formation for lateral meristems, primary leaf nodes and the cotyledonary nodes derived from 28-d old seedlings of different cotton cultivars.

	BA Concentrations		
	0.0 μM	0.3 μM	1.0 μM
HS-26	9[a]	83	68
	46[b]	83	79
CA-3050	40	75	50
	80	75	100
CA-3066	18	65	64
	73	88	100
CA-3076	55	100	86
	33	107	71
CA-3084	60	93	50
	80	100	60
Stovepipe	53	76	62
	71	71	62
Stoneville 474	47	44	44
	77	71	61

[a]The number of shootlets after two weeks/number of nodal meristems expressed as a percentage.

[b]The number of shootlets after three weeks/number of nodal meristems expressed as a percentage.

Table 8. Effect of Benzyladenine on shooting efficiencies of apical meristems excised from 14-21 day old seedlings of cottom (Stoneville 7A, glandless).

	Benzyladenine (μM)				
Culture period	0.0	0.15	0.3	0.5	1.0
Week 2	0/2[a](0/2)[b]	3/4 (0/4)	4/9 (0/9)	3/6 (0/6)	3/3 (0/3)
Week 3	2/2 (0/2)	2/4 (0/4)	3/8 (0/8)	4/6 (0/6)	3/3 (3/3)

[a]The number of shootlets/number of apical meristems.

[b]The number of elongated shootlets (> 2-3 cm tall)/number of apical meristems.

Table 9. Effect of Benzyladenine on shooting efficiencies of apical meristems excised from 14-21 day old seedlings of cottom (Paymaster HS-26, glanded).[†]

	Benzyladenine (μM)					
Culture period	0.0	0.15	0.3	0.5	1.0	10.0
Week 2	3/6[a](0/6)[b]	4/6 (0/6)	8/9 (0/9)	6/7 (0/7)	7/7 (0/7)	2/4 (0/4)
Week 3	1/8 (0/8)	6/7 (0/7)	9/9 (0/9)	4/7 (0/7)	6/8 (6/8)	0/4 (0/4)

[†]Similar data (not shown) has been collected for the following cultivars: CA-3076; CA-3050; CA-3066; CA-3084; and Stovepipe.

[a]The number of shootlets/number of apical meristems.

[b]The number of elongted shootlets (> 2-3 cm tall)/number of apical meristems.

Using the Shooting Medium as a screening system, different explant sources derived from *in vitro* grown cotton (Stoneville 7A) seedlings were tested for their ability to form shootlets (*See* Table 2). After a 21-day culture period, only cotyledonary nodes and primary leaf nodes formed shootlets; the remaining explants formed crystalline type callus primarily at the cut ends of the explant. Occasionally, a hypocotyl segment formed a shootlet which apparently developed from the pericycle region.

High shooting efficiencies occurred for both primary leaf nodes and cotyledonary nodes derived from 14 to 28-day old cotton seedlings when cultured on Shooting Medium containing 0.3 μM BA (Table 10). Explants from both the glandless variety (Stoneville 7A) (94%) and the glanded variety (Paymaster HS-26) (97%) possessed a prolific capability to form shootlets under these cultural conditions. Both the glanded and glandless varieties generally demonstrated multiple shootlet formation after about 3 weeks of culture.

Table 10. Shootlet Proliferation of Excised Primary Leaf Nodes and Cotyledonary Nodes from 14-Day Old Cotton Seedlings When Cultured on Shooting Medium Containing 0.3 μM BA.

Cultivar	Number of Explants	Number of Shootlets/Explants	% Explants Formed Shootlets	Time Required Shootlet Formation
Stoneville 7A	46	2-5	94	7-21 days[a]
Paymaster HS-26	37	2-5	97	7-21 days[a]

[a]Twenty-one days is required for the formation of elongated shootlets (2-3 cm), however, shootlet growth is generally first visible after about 7 days.

Rooting of the shootlets derived from the pre-existing meristems of clonally propagated shootlets, the nodal or apical meristems of 14- to 28-day old seedlings, or the apical meristems of 1 or 2-day old germinated seeds was accomplished in one of two ways. Shootlets were either cultured on Rooting Medium (Table 1) or rooted by transferring the shootlets directly to soil after treating the shootlet with Rootone™ (Table 11). Of the cultivars examined (Stoneville 7A and Paymaster HS-26), elongated shootlets demonstrated a low efficiency rate for root formation when cultured on MS medium plus 1 μM IBA. However, the glanded and glandless varieties (Paymaster HS-26 and Stoneville 7A) demonstrated a high efficiency for rooting when placed directly into soil after the application of Rootone™ (Table 11). Elongated shootlets of non-transformed Stoneville 7A and Paymaster HS-26 as well as KAN selected shootlets of Stoneville 7A, Paymaster HS-26, Sphinx, and CA-3076 have all been rooted successfully by advancing directly to soil (with Rootone™).

Table 11. Comparison of Rooting Efficiency of Cotton Shootlets When Cultured on Shooting Medium Containing Either 1 μM IBA or Rooted Directly by Transferring to Soil (+ Rootone™)

Cultivar	*In vitro* Culture Period MS + 1 μM IBA	Rooted Directly to Soil (+ Rootone ™)
Stoneville 7A	6/23[a]	12/23
Paymaster HS-26	6/27	25/28

[a] The number of shootlets that rooted / number of attempts.

To establish plantlets via rooting on the IBA supplemented Rooting Medium, the elongated shootlets were cultured on the Rooting Medium for about 6 weeks until rooting had occurred. The plantlets were then transferred to soil in 3 inch pots and hardened as described.

To establish plantlets via rooting directly in soil, the base of the elongated shootlets were first dipped into Rootone™ and then placed into 3" pots and hardened for 2 to 3 weeks. Once the roots were established, all regenerants and KAN selected plantlets began to elongate and develop new leaves. The plantlets were moved to 6 to 10 inch pots at the 4-leaf stage for plant maturation and flowering/seed set. The soil consisted of 3 parts potting soil and 1 part vermiculite. All regenerated plants that were advanced to soil were normal, and all of the matured plants regenerated to date have initiated flowers and set viable seed under greenroom conditions as previously described in Example 3. (Table 12). The establishment of plantlets via rooting in agar required approximately 3 months; however, complete plantlet formation by rooting directly in soil took half that time, i.e., approximately 6 weeks.

Table 12. Flowering and Seed Set of 137[a] Bolls From 32 Paymaster HS-26 Plants

Cultivar	% Germ	# Flowers	Seeds	# Germ
HS-26	73%	15	419	306
HS-26	74%	23	573	423
HS-26	65%	6	138	90
HS-26	69%	10	152	105
HS-26	88%	9	178	156
HS-26	84%	18	357	300
HS-26	72%	15	208	150
HS-26	87%	27	535	466
HS-26	71%	14	292	207
Totals	77%	137	2,852	2,203

[a]The 137 bolls generated a total 2,852 seeds for an average of 20.8 seeds/flower.

Example 5 - *Agrobacterium tumefaciens* Mediated Cotton Transformation Procedures

Explants consisting of meristematic tissue from 1 or 2-day old seedling (apices of isolated seed embryos) (Fig. 1a) and 14 to 28-day old seedlings (nodal meristems, i.e., leaf nodes and cotyledonary nodes) (Fig. 1b) were co-cultivated with *Agrobacterium tumefaciens* strain LBA4404 harboring the binary vector pBI121 ("Agro+") at a bacterial concentration of approximately 8 x 10^8 cells/ml. The binary vector pBI121 carries the selectable marker (NPT II gene) for kanamycin resistance and a GUS-reporter gene (Clontech Laboratories, Inc., Palo Alto, CA). As a control for each transformation experiment, LBA4404 without the binary vector pBI121 ("Agro-") was used. Bacteria were grown at 27°C in liquid LB (Luria-Bertani) medium (PH 6.6-7.0) and maintained in liquid medium with weekly transfers to fresh medium. For transformation experiments, bacteria were allowed to grow to an $O.D._{550}$ of approximately 1.7-2.0. Alternatively, an $O.D._{550}$ of approximately 0.1 to 0.8 is used. Appropriate antibiotics, streptomycin (25 μg/ml) and kanamycin (50 μg/ml) for Agro+ or streptomycin (25 μg/ml) for Agro- were present throughout the vegetative growth of the bacteria. Before transformation solid kanamycin (50 μg/ml) was added to the liquid culture (50 ml) of Agro-. Both were then diluted 1:1 during the co-cultivation phase.

Explants from the source materials were placed into 7 ml of the Co-cultivation Medium (Table 1) under sterile conditions. To this was added an equal volume of the LB-*Agrobacterium tumefaciens* strain LBA4404 solution. The explant - Co-cultivation Medium-LB-*Agrobacterium tumefacians* solution was then placed in a dessicator and subjected to a vacuum pressure (29" Hg) for about 5 minutes. The co-cultivation period consisted of a 15 minute period for temperature equilibrium (25°C or 30°C) and an incubation period of 1 hour under sterile conditions.

Example 6 - Transformation and Selection Procedures

Following the co-cultivation period, the explants were blotted on sterile filter paper and transferred to Shooting Medium plus antibiotics, i.e., a KAN Selection Medium for KAN selection. Alternatively, following the co-cultivation period, the explants are washed with LB medium + 500 μg/ml carbenicillin before they are blotted on sterile filter paper and transferred to KAN Selection Medium. The KAN selection procedure for the putatively transformed explants of the 1 or 2-day old and the 14- to 28-day old seedlings involved a cut/trim step procedure and a KAN 37 to 50 to 75 μg/ml or a KAN 37 to 50 μg/ml selection process, respectively. Essential to the success of the KAN selection procedure was the use of the cut/trim method in which the shootlets were cut and the cotyledonary leaves and other lateral nodes trimmed when necessary to constantly maintain the apical meristem less than 2 cm from the antibiotic source. This was done by evaluating the shootlets on a daily basis to determine their height. For the apical meristems of 1 or 2-day old seedlings, trimming was generally first performed after about 4 to 7 days. For nodal meristems of the 14 to 28-day old seedlings, trimming was generally first performed after about 7 to 10 days.

First, the co-cultivated explants were placed on Shooting Medium (0.3 μM BA) + 37 μg/ml KAN (+ 500 μg/ml carbenicillin) (KAN Selection Medium I - Table 1) to allow for shootlet formation. The developing explants were monitored daily for *Agrobacterium* contamination and elongated growth of either the hypocotyl or stem portion of the source materials utilized. In general, apices from 1 or 2-day old seedlings grew at a faster rate than the other explants, therefore, the hypocotyl of the germinating apices were usually cut/trimmed after about 5 days and, then, transferred to Shooting Medium (0.3 μM BA) + 50 μg/ml KAN + 500 μg/ml carbenicillin (KAN Selection Medium II - Table 1). In contrast, the developing shootlets derived from explants of 28-day old seedlings and clonally propagated shootlets grew at a slower rate and, therefore, were transferred to KAN Selection Medium II after about 7 days and were cut/trimmed when necessary. Following selection with KAN Selection Medium II, putative transgenic shootlets derived from cultured explants from 1 or 2-day old seedlings required an additional KAN selection pressure. These shootlets were therefore transferred to Shooting Medium (0.3 μM BA) + 75 μg/ml KAN + 500 μg/ml

carbenicillin (KAN Selection Medium III - Table 1) and were cut/trimmed when necessary. Using this cut/trim method, the developing explants (shootlets) were kept close (less than about 2 cm) to the kanamycin source for continual selection; also, the cutting kept the vascular tissues in direct contact with the MS medium (+ kanamycin).

The KAN-selection procedure described above involved a step-wise increment of 37 μg/ml KAN (5-7 days) to 50 μg/ml (4 weeks) to 75 μg/ml (4 weeks), each step containing carbenicillin at 500 μg/ml. In all cases, lateral meristems required only the first two levels (37 to 50 μg/ml) of kanamycin selection while apices from the 1 or 2-day old seedlings required the complete KAN-selection procedure (37 to 50 to 75 μg/ml). All developing shootlets subjected to this selection procedure were cut/trimmed as follows: hypocotyl/epicotyl from germinating seedlings were cut to 2 cm on a regular basis and the stems with lateral meristem growth were trimmed when necessary before transferring to the next medium combination. Shootlets that were advanced through this KAN-selection procedure [50 μg/ml (4 weeks) to 75 μg/ml (4 weeks)] were transferred on a weekly basis. Following a maturation step with Maturation Medium I or Maturation Medium II (Table 1), these KAN-selected shootlets (3-leaf stage) were rooted directly by transferring to soil (+ Rootone™). For germline transformation, various tissues such as pollen grains, leaf tissues and genomic DNA were harvested from maturing plants and assayed for GUS gene expression (fluorescent and histochemical localization of GUS activity) and DNA integration (Southern-blot analysis).

To verify kanamycin resistance, a number of shootlets from the CA-3076 and Paymaster HS-26 varieties were rechallenged on kanamycin rather than rooted. Explants of nodal and apical meristematic tissues were excised from these shootlets and cultured on fresh KAN Selection Medium II (Example 6) to identify transgenic meristems within these shootlets. All of the rechallenged apical meristems and most of the nodal meristems developed green shootlets.

Example 7 - Transformation and Selection Results

For transformation, explants from 14 to 28-day old seedlings (cotyledonary nodes, primary leaf nodes, nodal meristems, and apices) were used as starting materials with the regeneration system described herein (Table 13). Also used with this

regeneration system were apices (2 to 3 mm) excised from 1 or 2-day old seedlings or apical and nodal meristems from clonally propagated shootlets ("CPS"). (Table 13). Explants from both these sources were harvested and maintained for 1-2 hours in 7 ml of Co-cultivation Medium (Table 1) sterilized by filtration through a 0.22 μm Acrodisc filter (Gelman Sciences, Ann Arbor, Michigan).

Following excision of explants, an equal volume of LB-*Agrobacterium tumefaciens* strain LBA4404 containing pBI121 was added to Co-cultivation Medium/explants and incubated for approximately 1 hour at either 25° or 30°C. After the co-cultivation period, the explants were blotted and transferred onto the KAN selection procedure as described in Example 6. After two, 2-week culture periods on KAN Selection Medium II or on each of KAN Selection Medium II and III, the kanamycin-resistant shootlets elongated to approximately 2 to 3 cm, while the kanamycin-sensitive explants ceased to grow and died. (Table 13). Transformation efficiencies (based on the number of explants cultured) varied depending upon the genotype, explant source (Tables 13 and 14), and temperature (Table 14). In general, as indicated in Table 13, the 1-day system supported a higher regeneration/transformation efficiency rate (18%) than both the 28-day (11.9%) and CPS (1.4%) systems. However, based on explant and temperature (Table 14), Paymaster HS-26 (glanded) demonstrated explant-independency at both 25° and 30°C. In contrast, Stoneville 7A (glandless) demonstrated explant-dependency for both temperature and efficiency rates for regeneration/transformation (Table 14). In both cases, however, 25°C supported a higher regeneration/transformation efficiency rate than 30°C for co-cultivation (Table 14). Under greenroom conditions, the KAN selected plantlets matured to flowering stage and set seeds. Several KAN selected (T_0) plants expressed GUS enzymatic activity in leaves and pollen. (*See* Tables 15-19).

Table 13. Summary of KANAMYCIN-selected Shootlets and Mature Plants

Cultivar	Experiment#	# Explants	KAN-selected Shootlets			Mature Plants		
			1-day	28-day	CPS	1-day	28-day	CPS
CA-3076	#1	21	10			8		
HS-26	#1	28		8			6	
ST-7A	#4	42	24			16		
HS-26	#5	119		24			6	
HS-26	#8	48	8			-		
Sphinx[a]	#12	104	5			4		
ST-474	#14	62	2			-		
ST-139[b]	#15	65	1			-		
HS-26	#17	25		2		-		
ST-474[b]	#18	142			2			-
HS-26	#20	50	20			-		
ST-7A	#21	240		15			-	
Totals[c]		946	70	49	2	28	12	0

[a]Sphinx seeds were provided by Dr. K.M. El-Zik at Texas A&M Univ., College Station, Texas.

[b]Stoneville 474 and Stoneville 139 seeds were provided by Dr. Catherine Houck, Calgene, Davis, California.

[c]For apical meristematic explants from 1 or 2-day old germinating seeds, a total of 392 explants have been tested, 18% of which have generated shootlets. For nodal or apical meristematic explants from 14 to 28-day old seedlings, a total of 412 explants have been tested, 11% of which have generated shootlets. For meristematic explants of clonally propagated shootlets, a total of 142 explants have been tested, 1.4% of which have generated shootlets.

Table 14. Efficiency rate of KAN-selected shootlets when co-cultivated with *A. tumefaciens* at 25°C vs. 30°C. Pre-existing meristems from 1 or 2-day old germinated seeds and 28-day old seedling were used as starting material.

Cultivar	25°C KAN-selected	30°C KAN-selected
ST-7A[a]	12/17[c] (70%)	12/25 (48%)
ST-7A[b]	9/112 (8%)	6/128 (4.6%)
HS-26[a]	14/28 (50%)	6/22 (27%)
HS-26[b]	24/49 (48.9%)	17/58 (29%)

[a]Apices from 1-day old seedlings.

[b]Apices, lateral and cotyledonary meristems from 28-day old seedlings.

[c]Total number of KAN-selected shootlets/total number of pre-existing meristems.

Kanamycin-resistant shootlets obtained after transformation of apical meristematic tissue from 1 or 2-day old germinating seeds were of two types - green and mottled green. The green phenotype was exhibited by early emerging shootlets, which were generally evident after about five days and which did not show any phenotypic stress. The mottled green phenotype was exhibited by later emerging shootlets, which were generally evident after about 5 days to 2 weeks and which showed various degrees of stress such as bleached leaves, white leaf tips and edges, and green sectors and white vascular tissue in cotyledonary leaves. In addition, the mottled green phenotype grew more slowly during the KAN selection procedure (37, 50 and 75 μg/ml). However, newly formed leaves of the mottled green phenotype which emerged after about 2 weeks, were phenotypically normal and uniformly green. The KAN-resistant shootlets of both types were carefully moved through the previously described KAN selection procedure. Following selection, both the green and mottled green phenotypes were matured and rooted easily by the methods described herein. Kanamycin-sensitive explants did not exhibit proliferation; but rather, turned brown and died.

To date, 121 KAN-selected shootlets and 40 mature viable plants have been generated by this procedure with an efficiency range of 1.4 to 57% (Table 13). Under

greenroom conditions, these KAN-selected plants were morphologically normal; they flowered and developed viable pollen grains. As determined by the GUS assays (Jefferson 1987, Jefferson *et al.* 1987, Gallagher 1992, Stomp 1992), these KAN-selected plants (T_0) were found to have a chimeric growth pattern for positive GUS activity within their leaves and flower parts (pollen grains, pollen sacs and pollen grain tubes) (Tables 15-19).

The cotton cultivars used herein exhibited maximum selection at incremental kanamycin concentrations of 37 to 50 μg/ml for nodal and greater than 2-day old apical meristematic tissues and 37-50-75 μg/ml for 1 or 2-day old apical meristematic tissue. These kanomycin selection levels were selected using a conventional antibiotic screen. Other cultivars, however, may require different selection procedures or conditions. Appropriate selection conditions for additional genotypes can be easily determined by incorporating a "mork" transformation control -- *Agrobacterium tumefaciens* without the pBI121 binary vector (Agro-) -- and a stepwise incremental increase in KAN concentration. The level at which tissue or shoots co-cultivated with Agro- die is the optimum level of KAN to be used to select transformants using Agro+.

Example 8 - Introduction of Binary Vectors Into *Agrobacterium tumefacians*

DNA transfection of *Agrobacterium tumefacians* was accomplished by the triparental mating procedure. Ditta, et al., *Proc. Natl Acad. Sci. U.S.A.* 77:7347-7351 (1980); *Methods in Plant Mol. Biol.*, Cold Spring Harbor Laboratory Press pp. 63-65 (1995) (both of which are incorporated herein by reference). Two parental lines, *E. coli* HB101 - pRK2013 and *Agrobacterium* LBA4404, were obtained from Clontech Laboratories, Inc., Palo Alto, CA. The third parental line, *E. coli* HB101 *RecA*$^-$, was made competent and transformed (plasmid pBI121 - Clontech Laboratories, Inc.) by the calcium chloride procedure. Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, 2d ed, Cold Spring Harbor, 1:1.74 (1989). The *E. coli* HB101 *RecA*$^-$cells were provided by Dr. Dan Kunz (Department of Biological Sciences, University of North Texas, Denton, TX). These cells can also be purchased from Gibco BRL. (BRL Laboratories, Gaithersberg, MD).

Example 9 - Fluorometric Analyses of GUS Activity in Developing Shootlets

The fluorometric analysis of GUS activity was carried out as described by Jefferson (Jefferson, R.A., *Pt. Mol. Biol. Reporter* 5:387-405 (1987); Gallagher, S.R., *GUS Protocols*, Academic Press, Inc., pp. 47-59 (1992)) (both of which are incorporated herein by reference) using 4 mM 4-methylumbelliferyl-β-D-glucuronide (MUG) as the substrate except that 100 mM potassium phosphate buffer (pH 7.2) was used in the extraction buffer (100 mM KPO_4, pH 7.0; 10 mM β-mercaptoethanol; 10mM Na_2 EDTA; 0.1% sodium lauryl sarcosine and 0.1% Triton X-100). Insoluble polyvinylpyrrolidone (5%) and silicon powder was added to the extraction buffer (0.5 ml) before homogenization. Leaf tissue (approximately 20 mg of fresh weight) was homogenized in Eppendorf tubes by a motorized conical teflon pestle (1100 rpm) in 0.5 ml of the extraction buffer. Assays (GUS activity) of leaf extracts were performed on leaves formed after rooting. Green leaves from several nodes were also analyzed to identify chimeric plants. Readings were made on an Aminco Bowman Scanning Spectro-fluorometer (SLM Amino, Rochester, N.Y.) with a xenon lamp at excitation wavelength (365 nm) and emission wavelength (455 nm). GUS activity was measured at time intervals (0, 30, 60, 150 minutes and overnight) and expressed as relative fluorescence units/hour/μg protein. Representative results are shown in Table 15.

Table 15. Fluorometric Analysis of GUS Activity in KAN-selected Plantlets

Cultivar	Fluorescence units/hour/μg protein
HS-26NT-01[a]	0.99
HS-26NT-02	0.40
HS-26T0-01[b]	0.40
HS-26T0-02	0.60
HS-26T0-03	7.40
HS-26T0-04	3.54
HS-26T0-05	0.34
CA-3076T0-01	5.70
CA-3076T0-02	0.50
CA-3076T0-04	0.90
CA-3076T0-05	0.17

[a] "NT" refers to non-transformed plants.

[b] T0 (or T_0) indicates transformants.

Example 10 - Histochemical Analysis of GUS in Pollen Grains

A histochemical localization of GUS in the pollen of non-transformed cotton plants (HS-26NT-01) and in the transformed cotton plants whose leaves exhibited the greatest GUS activity (HS-26T0-04, CA-3076T0-01, HS-26T0-03) was performed according to the technique of Jefferson, R.A., *Plant Molecular Biology Reporter* 5(4):387-405 (1987) (incorporated herein by reference). The presence of insoluble, blue product inside pollen grains of transformed plants (Table 16) indicates germline transformation and transmission of the inserted GUS gene to gametes and subsequently to the progeny of the transformed plants produced by the methods discussed herein.

Example 11 - Histochemical localization of GUS activity in leaf tissue and flower parts

Leaves of shootlets and small plantlets and flower parts (pollen, pollen sac and pollen tubes) were tested for GUS expression as previously described (Jefferson 1987; Stomp 1992). The histochemical localization of GUS activity (Jefferson 1987, Stomp 1992) demonstrated that many of the KAN-selected plants generated the distinguishing blue color reaction in their pollen grains (HS-26T0's-Table 17; ST7AT0's-Table 18; CA-3076T0's-Table 19. T_0 plants appeared to be chimeric because some flowers produced GUS positive pollen while others did not. However, T_1 plants showed a much higher percentage of flowers with GUS positive pollen, indicating that they are not chimeric. The expression of GUS in pollen indicates germline transformation. (Table 19).

Table 16. Histochemical Analysis of GUS in Pollen Grains

Individual Plants	Expression of GUS
HS-26NT0-01 (nontransformant)	-
HS-26T0-03 (transformant)	+[a]
HS-26T0-04 (transformant)	+
CA-3076T0-01 (transformant)	+

[a]The (+) indicates the presence of precipitated enzyme reaction product inside pollen grains.

Table 17. Histochemical Localization of GUS Activity in Pollen Grains of Putative Transgenic Cotton Plants, c.v. Paymaster HS-26. Pre-existing meristems were excised from 28-day old seedlings and utilized as starting material for co-cultivation with *Agrobacterium*.

Individual Plants	Total # Flowers Tested	Total # Flowers w/+Pollen	Pollen in Sac (Pollentube)
HS-26T0-01[a]	9	3	1
HS-26T0-02	10	3	-
HS-26T0-03	6	5	-
HS-26T0-04	13	3	-
HS-26T0-05	12	2	-
HS-26T0-07B[b]	23	8	-
HS-26T0-08[b]	6	3	-(2:Pollentube)
HS-26T0-09	6	1	-
HS-26T0-11	3	-	-
HS-26T0-12	12	5	-
HS-26T0-13	9	4	1(1:Pollentube)
HS-26T0-14	23	11	-
HS-26T0-01:T1-03[c]	2	1	-
HS-26T0-01:T1-04	2	2	-
HS-26T0-01:T1-06	3	-	-
HS-26T0-01:T1-07	4	1	-
HS-26T0-01:T1-09	1	-	-
HS-26T0-03:T1-03	7	4	-(1:Pollentube)
HS-26T0-03:T1-44	2	2	-
HS-26T0-04:T1-04	6	4	-
HS-26T0-04:T1-08	2	1	-
HS-26T0-04:T1-23	2	1	-
HS-26T0-04:T1-29	1	1	-
HS-26T0-04B:T1-41	2	-	-

HS-26T0-04:T1-42	9	7	-(2:Pollentube)
HS-26T0-04:T1-43	1	1	-
HS-26T0-07B:T1-01	7	6	-
HS-26T0-07B:T1-08	1	1	-
HS-26T0-07B:T1-10	3	2	-
HS-26T0-07B:T1-12	2	2	1
HS-26T0-07B:T1-14	2	1	-
HS-26T0-07B:T1-40	6	6	-
HS-26T0-07B:T1-44	8	8	-

[a]T0 (or T_0) indicates transformants.

[b]A mottled green phenotype.

[c]T1 (or T_1) indicates progeny (seeds or plants) of T_0 plants. For example, HS-26T0-01:T1-03 designates one of several T_1 progenies of a T_0 plant.

Table 18. Histochemical Localization of GUS Activity in Pollen Grains of Putative Transgenic Cotton Plants, Stoneville 7A. Apices were excised from 1-day old seedlings and utilized as starting material for co-cultivation with *Agrobacterium*.

Individual Plants	Total # Flowers Tested	Total # Flowers w/+Pollen	Pollen in Sac (Pollentube)
ST7AT0-01	5	4	-
ST7AT0-02	3	1	-
ST7AT0-03	5	1	-
ST7AT0-04	7	3	-
ST7AT0-05	3	2	-(1:Sac)
ST7AT0-06	5	4	-(1:Pollentube)
ST7AT0-07	14	8	-
ST7AT0-08	19	8	-
ST7AT0-09	12	2	-
ST7AT0-10	10	3	-
ST7AT0-11	9	4	-
ST7AT0-12	7	2	-
ST7AT0-13	8	1	-(1:Pollentube)
ST7AT0-14	15	5	-(1:Pollentube)
ST7AT0-15	13	5	-(1:Pollentube)
ST7AT0-16	11	2	-(1:Pollentube)

Table 19. Histochemical Localization of GUS Activity in Pollen Grains of Putative Transgenic Cotton Plants, CA-3076. Apices were excised from 1-day old seedlings and utilized as starting material for co-cultivation with *Agrobacterium*.

Individual Plants	Total # Flowers Tested	Total # Flowers w/+Pollen	Pollen in Sac (Pollentube)
CA-3076T0-01	14	4	-
CA-3076T0-02	9	3	-
CA-3076T0-03	17	10	2*(1:Pollentube)
CA-3076T0-04	16	5	1(1:Pollentube)
CA-3076T0-09	2	-	-
CA-3076T0-10A[b]	1	1	1
CA-3076T0-11[b]	21	6	-
CA-3076T0-12[b]	2	1	-
CA-3076T0-01:T1-00[c]	16	7	-(1:Pollentube)
CA-3076T0-01:T1-01[c]	9	3	1
CA-3076T0-01:T1-02[c]	13	8	1
CA-3076T0-01:T1-03[c]	16	8	-
CA-3076T0-01:T1-04[c]	30	18	-(1:Pollentube)
CA-3076T0-01:T1-05[c]	18	11	-
CA-3076T0-01:T1-06[c]	19	14	-
CA-3076T0-01:T1-07[c]	21	12	-
CA-3076T0-01:T1-08[c]	20	11	-
CA-3076T0-01:T1-09[c]	10	2	-
CA-3076T0-01:T1-10	3	3	-
CA-3076T0-01:T1-11	3	3	1
CA-3076T0-01:T1-12	3	3	-
CA-3076T0-01:T1-13	1	-	-
CA-3076T0-01:T1-31	1	1	-

CA-3076T0-03:T1-30	1	1	-
CA-3076T0-04:T1-11	4	4	-
CA-3076T0-04:T1-13	1	1	-

[a] Sac stained positive while pollen grains were negative.

[b] A "mottled green" phenotype.

[c] No kanamycin selection of T_1 seeds.

Example 12 - Southern Blot Analysis of genomic DNA isolated from nuclei of cotton leaves

Cotton genomic DNA was extracted from isolated nuclei of fresh young leaves (Paterson *et al.* 1993). Purity and yield of DNA preparations were estimated spectrophotometrically (Sambrook *et al.* 1989). DNA (15 μg) was digested with 10 units/μl of Bam HI or Hind III and then electrophoresed in 0.8% agarose for 30 min at 20 volts and then 2 h at 60 volts. The genomic DNA was transferred to nylon membranes (Zeta-Probe by BioRad) for hybridization with a random primer-labelled GUS probe (provided by Dr. R. Pirtle's laboratory). The location of the bound GUS probe was identified by an alkaline phosphatase-catalyzed chemiluminescent reaction (*Gene Images* CDP-*Star* detection module, Amersham LIFE SCIENCE).

The GUS gene was shown to be incorporated into the cotton genome of the T_0 plants, i.e., the transformants, by Southern blot analyses. Following digestion with Hind III and subsequent resolution by electrophoreses, the GUS probe hybridized to a genomic DNA band at approximately 9 kb for the HS-26T_0-03 plant and a corresponding band was not found for the HS-26NT (nontransformed) plant. This was smaller than the linearized pBI121 vector (13 kb) indicating that the GUS fragment was not in the free plasmid or in *Agrobacterium*, but rather was integrated into the genomic DNA of the cotton plant.

Example 13 - In vitro T1 Seedling Assay

T_1 seeds from individual flowers of the T_0 plants were germinated in the presence of 50 KAN (50 μg/ml) to screen for the KAN-selected seeds of the next generation. The T_1 seeds expressing KAN-resistant capability appeared normal in comparison to the non-transformed T_1 seeds which showed poor germination with yellow root tips and swollen roots without extensive root hair development. The nontransformed control (HS-26NT) also showed these stressed characteristics (Data not shown). KAN-resistant T_1 seeds were transferred to soil and three phenotype growth patterns were evident in the T_1 plants after a 21-day growth period. These phenotypes were as follows: Large (epicotyl growth of 15 cm in height), Medium (7 cm in height) and Small (little or no epicotyl development - these usually died). Seeds within each boll and from different

bolls of the same T_0 plant showed different seed germination rates and seedling phenotpyes which is consistent with the concept that these T_0 plants are chimeric. As demonstrated in Tables 17, 18 and 19, these KAN-selected T_1 plants also were found to be GUS positive by the pollen assay. This strategy allows the rapid screening of T_0 and T_1 progeny to identify likely transformants.

Example 14 - Transgenic Screens

Selected individual plants have been tabulated to demonstrate the evaulation procedure utilized to describe the transgenic T_0 and T_1 plants (Table 20). The individual plants were scored (1 to 6) as described in Table 20. In brief, all the T_0 were shootlet KAN-selected and all the T_1 were seedling KAN-selected for the L phenotype. All putative transgenics had positive GUS activity for pollen. In most cases, these plants also scored high for fluorescent GUS activity for leaf protein and histochemical GUS activity for leaf mid-vein/petiole explants. To date, HS-26T0-03 showed a positive Southern test.

Table 20. Summary of transgenic screens: shootlet KAN-selection procedure for T_0s (1), GUS positive in the leaf fluorescence assay for T_0s and T_1s (2), GUS positive in the leaf mid-vein/petiole histochemical assay for T_0s and T_1s (3), GUS positive in the pollen histochemical assay for T_0s and T_1s (4), seedling KAN-selection procedure for T_1s: Large (L), Medium (M) and Small (S) plant development phenotypes (5) and Southern blot assay (6).

Individual Plants	(1)	(2)	(3)	(4)	(5)	(6)
HS-26T0-03	+	+	nd	+	na	+
HS-26T0-03:T1-03	na	+	-	+	L	nd
HS-26T0-04	+	+	nd	+	na	nd
HS-26T0-04:T1-04	na	-	+	+	L	nd
CA-3076T0-01	+	+	nd	+	na	nd
HS-26T0-01	+	nd	nd	+	na	nd
HS-26T0-01:T1-07	na	+	+	+	L	nd
HS-26T0-07B	+	nd	nd	+	na	nd
HS-26T0-07B:T1-08	na	-	+	+	L	nd
HS-26T0-07B:T1-16	na	+	+	+	L	nd

nd - not determined.

na - not applicable to the criteria in question.

WE CLAIM:

1. A method for regenerating cotton plants comprising the steps of:

(a) isolating an explant of a cotton plant wherein said explant comprises a pre-existing meristem;

(b) inducing the proliferation of a shootlet from the explant by culturing said explant with a nutrient media supplemented with a concentration of cytokinin which promotes shootlet proliferation; and

(c) rooting the shootlet.

2. The method of claim 1 wherein the pre-existing meristem is isolated from a seedling which is less than about 28 days old.

3. The method of claim 1 wherein the pre-existing meristem is isolated from a nodal or apical meristem of a seedling about 14 to about 28 days old.

4. The method of claim 1 wherein the pre-existing meristem is isolated from an apical meristem of a seedling about 1 or 2 days old.

5. The method of claim 3 wherein the nodal meristematic tissue comprises leaf nodes and cotyledonary nodes.

6. The method of claim 1 wherein the cytokinin is benzyladenine.

7. The method of claim 6 wherein the concentration of benzyladenine is no greater than about 1.0 μM.

8. The method of claim 6 wherein the concentration of benzyladenine ranges from about 0.15 μM to no greater than about 1.0 μM.

9. The method of claim 8 wherein the concentration of benzyladenine is about 0.3 μM.

10. The method of claim 1 further comprising the step of maturing the shootlet prior to rooting.

11. The method of claim 1 further comprising the steps of:

(d) isolating an explant from a first shootlet wherein said explant comprises a pre-existing meristem;

(e) inducing the proliferation of a second shootlet from the explant of the first shootlet by culturing said explant with a nutrient media supplemented with a concentration of cytokinin which promotes shootlet proliferation; and

(f) rooting the second shootlet(s).

12. A cotton plant produced by the method of claim 1.

13. A cotton seed produced by the plant of claim 12.

14. A cotton plant germinated from the seed of claim 13.

15. A method for genetically engineering cotton plants comprising the steps of:

(a) isolating an explant of a cotton plant wherein said explant comprises a pre-existing meristem;

(b) transforming the explant;

(c) inducing the proliferation of a shootlet from the explant by culturing said explant with a nutrient media supplemented with a concentration of cytokinin which promotes shootlet proliferation and selecting for a transformant in the presence of a selecting agent; and

(d) rooting the shootlet.

16. The method of claim 15 wherein the explant is transformed by the introduction of foreign DNA via *Agrobacterium tumefacians*.

17. The method of claim 16 wherein the explant is transformed by co-cultivating the explant with a culture of *Agrobacterium tumefacians*, said *Agrobacterium tumefacians* being transformation competent and harboring a plasmid comprising both a foreign gene and a selection agent resistance gene, both genes including appropriate regulatory sequences so as to be expressed in the cells of the cotton plant.

18. The method of claim 17 wherein the selection agent is an antibiotic and the resistance gene codes for antibiotic resistance.

19. The method of claim 18 wherein the antibiotic is kanamycin and the resistance gene is the NPTII gene.

20. The method of claim 19 wherein the pre-existing meristem is isolated from a seedling which is less than about 28 days old.

21. The method of claim 15 wherein the pre-existing meristem is isolated from a nodal or apical meristem of a seedling about 14 to about 28 days old.

22. The method of claim 15 wherein the pre-existing meristem is isolated from an apical meristem of a seedling about 1 or 2 days old.

23. The method of claim 21 wherein the nodal meristematic tissue comprises leaf nodes and cotyledonary nodes.

24. The method of claim 15 wherein the cytokinin is benzyladenine.

25. The method of claim 24 wherein the concentration of benzyladenine is no greater than about 1.0 μM.

26. The method of claim 25 wherein the concentration of benzyladenine ranges from about 0.15 μM to no greater than about 1.0 μM.

27. The method of claim 26 wherein the concentration of benzyladenine is about 0.3 μM.

28. The method of claim 15 further comprising the step of maturing the shootlet prior to rooting.

29. The method of claim 15 further comprising the steps of:

(d) isolating an explant from a first shootlet wherein said explant comprises a pre-existing meristem;

(e) inducing the proliferation of a second shootlet from the explant of the first shootlet by culturing said explant with a nutrient media supplemented with a concentration of cytokinin which promotes shootlet proliferation; and

(f) rooting the second shootlet.

30. A cotton plant produced by the method of claim 15.

31. A cotton seed produced by the cotton plant of claim 30.

32. A cotton plant germinated from the seed of claim 31.

33. A cotton plant produced by the method of claim 17.

34. The cotton plant of claim 33 comprising a genome in to which the foreign gene and the selection agent resistance gene have been inserted, wherein said plant produces a foreign cellular product encoded by the foreign gene.

35. A cotton seed produced by the cotton plant of claim 33.

36. A cotton plant germinated from the seed of claim 35.

37. A cotton plant which is the progeny of a cotton plant produced by the method of claim 15.

38. A cotton seed produced by the cotton plant of claim 38.

FIG. 1b

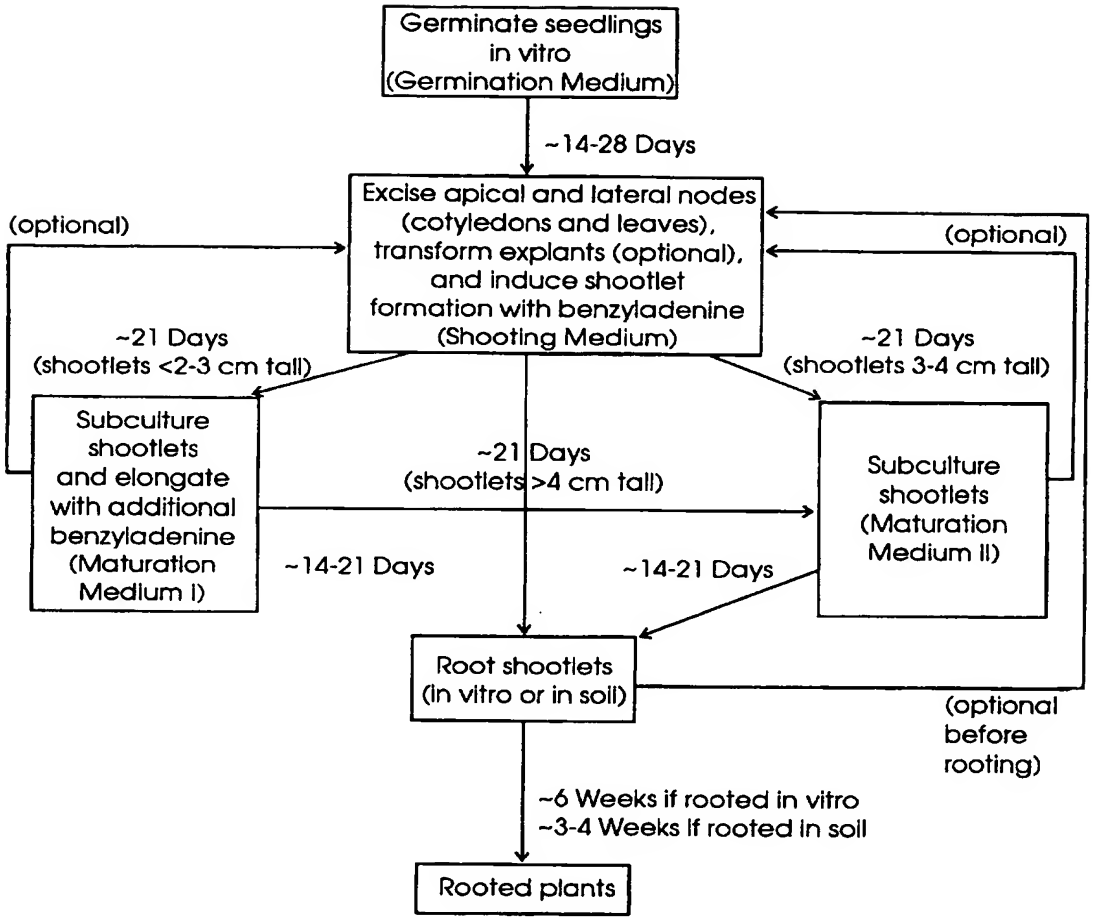
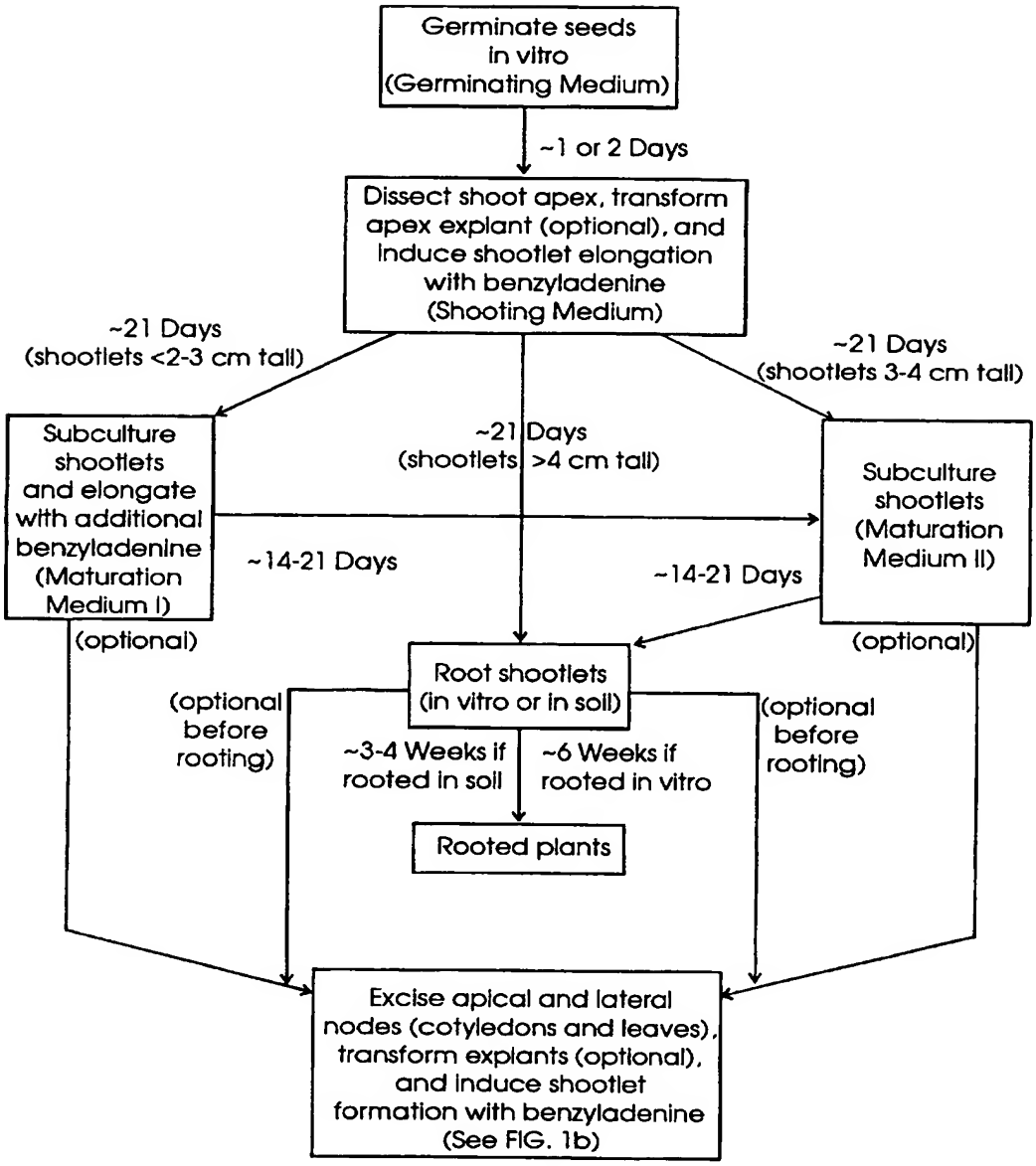

Germinate seedlings in vitro (Germination Medium)
~14-28 Days
Excise apical and lateral nodes (cotyledons and leaves), transform explants (optional), and induce shootlet formation with benzyladenine (Shooting Medium)
(optional)
(optional)
~21 Days (shootlets <2-3 cm tall)
~21 Days (shootlets 3-4 cm tall)
Subculture shootlets and elongate with additional benzyladenine (Maturation Medium I)
~21 Days (shootlets >4 cm tall)
Subculture shootlets (Maturation Medium II)
~14-21 Days
~14-21 Days
Root shootlets (in vitro or in soil)
(optional before rooting)
~6 Weeks if rooted in vitro
~3-4 Weeks if rooted in soil
Rooted plants

FIG. 1a


Germinate seeds in vitro (Germinating Medium)
~1 or 2 Days
Dissect shoot apex, transform apex explant (optional), and induce shootlet elongation with benzyladenine (Shooting Medium)
~21 Days (shootlets <2-3 cm tall)
~21 Days (shootlets >4 cm tall)
~21 Days (shootlets 3-4 cm tall)
Subculture shootlets and elongate with additional benzyladenine (Maturation Medium I)
~14-21 Days
(optional)
Subculture shootlets (Maturation Medium II)
~14-21 Days
(optional)
Root shootlets (in vitro or in soil)
(optional before rooting)
(optional before rooting)
~3-4 Weeks if rooted in soil
~6 Weeks if rooted in vitro
Rooted plants
Excise apical and lateral nodes (cotyledons and leaves), transform explants (optional), and induce shootlet formation with benzyladenine (See FIG. 1b)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/08242

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 A01H4/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A01H C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHLAN, C.A., ET AL. : "A PROCEDURE FOR BIOLISTIC TRANSFORMATION AND REGENERATION OF TRANSGENIC COTTON FROM MERISTEMATIC TISSUE" PLANT MOLECULAR BIOLOGY REPORTER, vol. 13, no. 1, 1995, pages 31-37, XP002041977 especially page 33, page 35, lines 9-15 see the whole document ---	1-7,10, 12-15, 21-25, 28,30-32
X	WO 95 06741 A (BIOCEM ;KNITTEL NATHALIE (AU); LENEE PHILIPPE (FR)) 9 March 1995	1-9, 12-27, 30-38
Y	pages 1,4,11,15,29 --- -/--	1-38

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 September 1997	15. 10. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Holtorf, S

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/08242

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 164 310 A (SMITH ROBERTA H ET AL) 17 November 1992 see the whole document	1-38
X	WO 92 15675 A (AGRACETUS) 17 September 1992 page 3,line 35-40; page 4,line 25-33; page 6,line 31-40; page 8, line 23-40, page 20, line 30	1-6,10, 12-15, 21-24, 28, 30-32,37
X	TRIPATHY,S., ET AL . : "INDUCTION OF DIRECT MULTIPLE SHOOTS FROM COTYLEDONS AND MERISTEMS OF GOSSYPIUM HIRSUTUM L." IN VITRO, vol. 31, no. 3, March 1995, page P-1057 XP002041382 see the whole document	1-4,6, 10,12-14
X	BAJAJ, Y., ET AL .: "MICROPROPAGATION AND GERMPLASM PRESERVATION OF COTTON (GOSSYPIUM spp.) THROUGH SHOOT TIP AND MERISTEM CULTURE" INDIAN JOURNAL OF EXPERIMENTAL BIOLOGY, vol. 24, September 1986, pages 581-583, XP002041383 see the whole document	1-6, 10-14
A	GOULD, J., ET AL .: "REGENERATION OF GOSSYPIUM HIRSUTUM AND G. BARBADENSE FROM SHOOT APEX TISSUES FOR TRANSFORMATION" PLANT CELL REPORTS, vol. 10, 1991, pages 12-16, XP002041978 see the whole document	1-38
A	US 4 992 375 A (WRIGHT MARTHA S) 12 February 1991 cited in the application see the whole document	1-38
P,X P,Y	WO 96 39021 A (MONSANTO CO) 12 December 1996 pages 12,13,34-39	1-6, 10-17, 28,30-38 1,8, 19-24
P,X	WO 96 26639 A (CALGENE INC) 6 September 1996 see the whole document	1-4, 12-17, 30-38

-/--

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

national Application No
PCT/US 97/08242

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	AGRAWAL, D.C., ET AL . : "IN VITRO INDUCTION OF MULTIPLE SHOOTS AND PLANT REGENERATION IN COTTON (GOSSYPIUM HIRSUTUM L.)" PLANT CELL REPORTS, vol. 16, no. 9, June 1997, pages 647-652, XP002041979 see the whole document -----	1-38

7

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Ir national application No.
PCT/US 97/08242

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 38
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

Claim 38 was read as being a cotton seed, produced by the cotton plant of claim 37.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 97/08242

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9506741 A	09-03-95	FR 2709496 A	10-03-95
		AU 7539694 A	22-03-95
		EP 0716706 A	19-06-96
		HU 74664 A	28-01-97
		SK 25896 A	04-09-96
US 5164310 A	17-11-92	AU 3756889 A	05-01-90
		CN 1042638 A	06-06-90
		DK 285590 A	31-01-91
		EP 0419533 A	03-04-91
		IE 65516 B	01-11-95
		IL 90440 A	16-10-96
		JP 3504800 T	24-10-91
		PT 90709 B	18-07-95
		WO 8912102 A	14-12-89
WO 9215675 A	17-09-92	AU 1663392 A	06-10-92
		AU 2161195 A	28-09-95
		CA 2082262 A	07-09-92
		EP 0531506 A	17-03-93
US 4992375 A	12-02-91	CA 1268631 A	08-05-90
WO 9639021 A	12-12-96	AU 6100096 A	24-12-96
		EP 0777412 A	11-06-97
WO 9626639 A	06-09-96	AU 5363296 A	18-09-96
		EP 0758841 A	26-02-97